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# STUDIES IN A RAT LUNG TUMOR MODEL: CELLULAR BIOCHEMISTRY AND CYTOGENETICS

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Roger C. Inman

ROGER C. INMAN, Colonel, USAF, BSC
Chief, Toxic Hazards Division

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An animal lung tumor model employing intratracheal inoculation of rats with a suspension of 3-methylcholanthrene (MCA) has been used to study induction of $^{3}$H-benzo(a)pyrene ($^{3}$H-BaP) metabolism in lung and liver, and chromosome damage in lung cells. A single inoculation with 1.0 mg MCA increased the enzyme levels in both lung and liver. The enzyme activity in the lung remained above control levels for 8 weeks, and in the liver for more than 2 weeks. Inoculation with 0.1 mg MCA was less effective in both organs.

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Sister chromatid exchange (SCE) incidence was measured in primary cultures of lung cells from MCA-treated rats. An increased SCE incidence was seen 48 hr posttreatment which persisted for at least 6 weeks after a single treatment with 1.0 mg MCA.

Tests for induction of DNA repair synthesis in vivo by MCA (1.0 mg) did not detect evidence of DNA repair.

## PREFACE

This is the second annual report of the Cytology, Cell Biology, and Cytogenetics Section of the Toxic Hazards Research Program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under Contract Number F33615-80-C-0512. This report describes the research activities at UCI from July 1, 1981 through June 30, 1982. During this period, T.T. Crocker, M.D. was Principal Investigator for the Contract. Ronald E. Rasmussen, Ph.D., conducted the studies at UCI. Jean Anderson served as Staff Research Associate, and Arthur Fong as Research Assistant. Technical Monitor for the Air Force was M.K. Pinkerton, AFAMRL/THT, WPAFB, Ohio.

During the period covered by this report, basic studies were conducted to determine some of the biological changes which occur when rats are treated intratracheally with a known lung carcinogen. This Section is concerned specifically with cytogenetic damage in lung cells, and the alterations in carcinogenic chemical metabolism caused by the treatment.

The goal of the studies is to obtain information on the biologic changes which accompany cancer initiation and growth in order to aid in the evaluation of test materials whose carcinogenic effects may be difficult to assess.

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## INTRODUCTION

### Statement of the Problem

Tests for the carcinogenic potential of chemicals usually have as their endpoint the actual development of tumors in experimental animals. However, it is well known that there are other effects of test agents which may or may not have relevance to carcinogenesis. Among these are chromosomal damage, DNA damage, enzyme induction, stimulation of cell proliferation in target organs or tissues, and effects on the immune response. In order to obtain information on the relationships of these effects to the complex process of tumorigenesis, a systematic study of chemically- mediated tumor initiation and growth is required.

### Specific Aims

An experimental animal model in which lung tumors are induced by the intratracheal inoculation of carcinogens is being used to study changes in selected factors during initiation and growth of malignant lung tumors. The animal model is the Fischer 344 male rat, which has been used in many studies of chemical carcinogenesis, and is susceptible to induction of lung carcinomas by treatment with the polynuclear aromatic hydrocarbon, 3-methylcholanthrene (MCA).

The specific factors measured were DNA replication and repair in lung tissue, $^{3}$H-benzo(a)pyrene ($^{3}$H-BaP) metabolism in lung and liver, and chromosomal damage in lung cells as indicated by increased incidence of sister chromatid exchanges (SCEs).

The work reported here is concerned with changes in these factors which take place during the initiation phase of tumorigenesis. Future studies will be done with animals carrying growing tumors, and with animals in which the tumors have grown to the point of lethality.

## MATERIALS AND METHODS

### The Animal Model

The induction of bronchiolo-alveolar squamous cell carcinomas in Fischer 344 rats by intratracheal inoculation with suspensions of MCA was reported by Schreiber et al. in 1972. This model offers a situation in which tumors can be induced in nearly all animals within a relatively short latent period. Further, the time of appearance of the tumors is dose-dependent. In the past year, experiments at the Toxic Hazards Research Unit (THRU) at WPAFB have confirmed the work of Schreiber et al. Inoculation of rats with suspensions of MCA

crystals at biweekly intervals has produced malignant lung tumors in rats receiving 5 treatments of 5 mg MCA each. In work at UCI, the methods of MCA administration are the same as those used at the THRU, although the rats are not held for tumor development.

Administration of MCA followed the procedures described by Schreiber et al. (1972) and Henry et al. (1981). The crystalline material was suspended in 0.2% gelation in 0.9% NaCl, and maintained in suspension by continuous stirring on a magnetic stirrer. Rats were anesthetized with methoxyflurane (Metofane) by placing the rats in a chamber through which a stream of air carrying the vapor was passed. For inoculation, a specially-designed speculum was used to hold open the rat's mouth, and a Teflon tube attached to a syringe containing the MCA suspension was passed into the trachea to about the level of the bifurcation, at which point the suspension was injected into the lung. At this writing, over 200 rats have been inoculated in this way with no significant trauma and no mortality due to the treatment method.

DNA Replication and Repair Synthesis in the Lung

The methods for this assay have been reported in detail previously (Rasmussen, 1981). In brief, the lungs were inflated *in situ* with warm, melted agar dissolved in a nutrient medium. After the agar was set, the lungs were sliced to pieces having a thinness of about 1 mm. The slices were incubated with $^{3}$H-thymidine ($^{3}$H-dThd, 5 μCi/ml) for 2-4 hours and the incorporation of label into DNA was determined using a chemical extraction method (Scott et al., 1956). The amount of DNA replication was calculated as $^{3}$H-dpm per microgram of DNA. For measurement of DNA repair synthesis, an inhibitor of replicative synthesis, hydroxyurea (HU, Brandt et al., 1972) was present in the incubation mixture at $10^{-2}$ M. This chemical inhibits normal semiconservative DNA replication, but at the concentration used does not inhibit repair synthesis. DNA repair synthesis was calculated by subtracting the incorporation of $^{3}$H-dThd into DNA of lung slices from control rats from the incorporation into DNA of slices from MCA-treated rats. DNA damage and subsequent repair was induced in lung slices by the addition of $10^{-3}$ M methylmethane sulfonate (MMS) to the incubation medium. MMS is a direct-acting aklylating agent, and was used to compare the DNA repair capacity between control and MCA-treated rats.

$^{3}$H-BaP Metabolism by Lung and Liver Microsomes.

The methods employed have been published (Rasmussen and Wang, 1974). In brief, the tissues from like-treated rats were pooled and homogenized using a Teflon-glass homogenizer, and the homogenate centrifuged at 9000 x *g* for 30 min to remove nuclei and

mitochondria. The supernatant was removed and centrifuged at 100,000 x g for one hour to obtain the microsomes. The microsomal pellet was resuspended in tris-sucrose buffer, and centrifuged a second time at 100,000 x g. The pellet was resuspended in a volume equal to the original weight of the tissue and protein content determined by the Lowry method. The incubation mixtures contained 0.5 mg protein, 1.8 mg NADPH, 20 μCi $^3$H-BaP (2.0 μg) in a final volume of 2.0 ml made up with SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.2). Incubation was for 30 min at 37° in air, with gentle shaking. The reaction was stopped by the addition of 4.0 ml of ethyl acetate and chilling in ice. After vigorous shaking, the ethyl acetate phase was removed, and the aqueous layer extracted a second time with ethyl acetate which was added to the first extract. The organic extracts which contained the hydroxylated metabolites of $^3$H-BaP as well as unchanged BaP were taken to near dryness under $N_2$ and the residue chromatographed on thin layer mylar-backed silica gel plates. Authentic nonradioactive derivatives of BaP were added to the samples to serve as markers on the TLC plates. The locations of the metabolites were visualized by examination of the plates with a UV lamp and by scanning of the plates with a radiochromatogram scanner. The metabolites were quantified by cutting the TLC plates into strips carrying the individual metabolites and counting in a scintillation spectrometer. Metabolite yields were calculated as picomoles/mg protein.

## Measurement of Cytochrome P-450 in Lung and Liver Microsomes

Spectrophotometric determination of cytochrome P-450 was done by an adaptation of the method of Omura and Sato (1964). Microsomes were suspended in tris-sucrose buffer, pH 7.2-7.4. Protein concentrations were adjusted to 2 mg/ml for liver microsomes and 3 mg/ml for the lung microsomes. Portions of 1 ml were placed in the cuvettes of a Varian/Cary dual beam spectrophotometer (Model 210), and a small amount of sodium dithionite added to reduce the iron of the cytochrome. After recording a baseline from 440-500 nm, one cuvette was flushed with analytic grade carbon monoxide (CO) to form the P-450-CO complex which has a characteristic absorption peak in the region of 450 nm. The difference spectrum was then recorded from 500 to 440 nm.

## Preparation of Primary Cultures of Lung Cells for SCE Analysis

During the present year methods have been developed for preparation of primary cultures of lung cells and for obtaining sufficient numbers of complete metaphase cells for SCE analysis. Using aseptic technique, the thoracic cavity of a phenobarbital-anesthetized rat was opened and the lung vasculature perfused with heparinized saline via the pulmonary artery. The required sample of

lung tissue was removed, minced to fragments of about 2 $mm^3$, and transferred to an enzyme solution containing 1% bovine serum albumin, thermolysin (750 U/ml) (Frazier et al., 1975) and collagenase (25 U/ml) in tris-buffered Hank's balanced salt solution, pH 8.0. After digestion for 1 hr at room temperature, the supernatant fluid was removed and replaced with a fresh portion of enzyme, and digestion continued for an additional hour. The released cells were harvested by centrifugation, counted, and 1-3 x $10^6$ cells seeded into 25 $cm^2$ plastic flasks with Waymouth's MB 752/1 medium supplemented with 10% heat inactivated fetal calf serum, 0.1 µg/ml hydrocortisone, 1.0 µg/ml insulin, and 100 µg/ml gentamicin. After incubation for 24 hr, the medium was removed and the adherent cells washed with fresh medium. For SCE analysis, BrdU ($10^{-5}$ M) was added at this time and the cells harvested 44-48 hours later. The methods used for SCE analysis were essentially those described by Latt et al. (1979, 1981) and Perry and Wolff (1974). Colcemid (0.1 µg/ml) was added 3 hr before harvest. The cells were released by trypsin, treated with 0.075 M KCl for 8 min, and fixed with methanol:acetic acid (3:1) for at least 2 hr. Slides were prepared and stained with Hoechst 33258 and Giemsa as described (Latt et al., 1982). Addition of the BrdU may be delayed until the 4th day after culture, but not much later since the cultures approach confluence on about the sixth or seventh day and the mitotic fraction declines greatly.

## EXPERIMENTAL RESULTS

### Effect of MCA Treatment on DNA Replication and Repair Synthesis in the Lung

In these experiments, MCA was administered to Metofane-anesthetized rats by making a small incision in the front of the neck and injecting the MCA suspension into the lower trachea between the cartilage rings. In subsequent experiments, this method was abandoned in favor of the less traumatic method described above under Materials and Methods. The dose of MCA was adjusted in each experiment to 5 mg MCA/kg b.w.

The results of these experiments are summarized in Tables 1 and 2. In both experiments, there was a slight reduction in the level of DNA replication in lung slices from MCA-treated rats compared in each case to the vehicle-treated controls. There was no indication in either experiment that MCA itself caused sufficient DNA damage in the lung so that repair synthesis could be detected (Compare values obtained when HU was present between vehicle and MCA-treated). When the lung slices were treated in vitro with MMS in order to cause DNA damage and to induce repair synthesis, the slices from MCA-treated rats showed slightly lower levels of DNA repair synthesis, again compared to the vehicle controls.

Table 1

DNA Replication and Repair Synthesis in Lung Slices from Rats Inoculated Intratracheally with MCA

Lung slices were incubated for 4 hr at 37°C with the indicated additions. The values are $^3$H-dpm per microgram of DNA, and are based on triplicate samples for each rat.

| | $^3$H-dThd Only | $^3$H-dThd + $10^{-2}$M HU | $^3$H-dThd + HU + $10^{-3}$M MMS | Calculated[a] DNA Repair |
|---|---|---|---|---|
| Untreated #1 | 3403 ± 105 | 229 ± 15 | 718 ± 46 | 489 ± 34 |
| Untreated #2 | 2437 ± 118 | 184 ± 20 | 588 ± 26 | 404 ± 23 |
| Anesthetized #1 | 2368 ± 396 | 224 ± 9 | 601 ± 45 | 377 ± 32 |
| Anesthetized #2 | 2137 ± 138 | 203 ± 12 | 608 ± 69 | 405 ± 50 |
| Sham-Operated #1 | 3111 ± 205 | 194 ± 23 | 595 ± 52 | 401 ± 40 |
| Sham-Operated #2 | 2257 ± 136 | 205 ± 40 | 574 ± 35 | 369 ± 38 |
| Vehicle #1 | 3309 ± 363 | 251 ± 30 | 536 ± 45 | 285 ± 38 |
| Vehicle #2 | 3943 ± 253 | 245 ± 34 | 652 ± 58 | 407 ± 48 |
| MCA #1 | 1156 ± 39 | 167 ± 17 | 393 ± 37 | 226 ± 29 |
| MCA #2 | 2519 ± 146 | 241 ± 38 | 571 ± 92 | 330 ± 70 |

[a] Column 3 minus Column 2.

Table 2

DNA Replication and Repair Synthesis in Lungs of MCA-Treated Rats Killed at 48 Hours

Treatment and values are as in Table 1.

| Treatment | $^3$H-dThd Only | $^3$H-dThd +$10^{-2}$M HU | $^3$H-dThd + HU + $10^{-3}$M MMS | Calculated[a] DNA Repair |
|---|---|---|---|---|
| Untreated #1 | 2502 ± 122 | 215 ± 17 | 576 ± 19 | 361 ± 18 |
| Untreated #2 | 2347 ± 236 | 171 ± 35 | 461 ± 36 | 290 ± 36 |
| Anesthetized #1 | 3216 ± 220 | 208 ± 12 | 546 ± 106 | 338 ± 75 |
| Anesthetized #2 | 8251 ± 700 | 309 ± 61 | 701 ± 79 | 392 ± 71 |
| Sham-Operated #1 | 2669 ± 271 | 189 ± 15 | 514 ± 50 | 325 ± 37 |
| Sham-Operated #2 | 1954 ± 412 | 171 ± 30 | 465 ± 33 | 294 ± 32 |
| Vehicle #1 | 1396 ± 176 | 163 ± 26 | 546 ± 25 | 383 ± 26 |
| Vehicle #2 | 5261 ± 988 | 184 ± 15 | 596 ± 121 | 412 ± 86 |
| MCA #1 | 1244 ± 347 | 173 ± 14 | 347 ± 47 | 174 ± 35 |
| MCA #2 | 1103 ± 182 | 137 ± 9 | 464 ± 31 | 327 ± 23 |

[a]Column 3 minus column 2.

Overall, the effect of intratracheal injection of MCA on DNA replication in the rat lung seemed to be minimal. Whether certain cell groups may have been affected differently will be the subject of future studies.

## Effect of MCA Treatment on In Vitro $^{3}$H-BaP Metabolism

Intratracheal injection of a suspension of MCA crystals induced the metabolism of $^{3}$H-BaP in both lung and liver. Further, the enzyme levels remained elevated for extended periods after a single treatment. The initial experiments used the inoculation method described above for the DNA replication studies. A special point was that the suspension of MCA crystals had been sonicated to reduce the crystal size to about 1 μm. In subsequent studies (see below) MCA preparations contained much larger crystals. The results of the first experiments are shown in Table 3. At 48 hr posttreatment, $^{3}$H-BaP metabolism by lung and liver microsomes from MCA-treated rats was clearly much increased over the vehicle controls. At one week posttreatment, the enzyme activities were still substantially higher in the microsomes from the treated rats. An experiment was done which extended for 3 weeks posttreatment, and the results are shown in Table 4. As before, the enzyme activities from microsomes from treated rats remained elevated at 7 days, but by 14 days had returned to near control levels.

## Effect of Particle Size of MCA on $^{3}$H-BaP Metabolism

At the THRU, an experiment had been initiated in early 1981 with the aim of establishing the dose of MCA required to produce lung tumors in rats within a specified time. The MCA preparation was similar to that used at UCI, and consisted of very small particles of MCA. By October, 1981, it was clear that no lung tumors had been induced in the rats at the THRU. Consultation with other investigators suggested that the particle size might be critical for the successful initiation of tumors. This has since proven to be the case. Rats inoculated with large particles of MCA in January, 1982, had developed malignant lung tumors by May. The nature of the 2 different preparations is illustrated in Figure 1. In MCA preparations subjected to prolonged sonication, the particles were all about 1 μm or less. The large particle suspension was prepared by recrystallizing the MCA from benzene, and then lightly grinding the crystals in an agate mortar with a little 0.9% NaCl just to the point that the crystals could be suspended long enough to permit the intratracheal injection. This method was used by Henry et al. (1981) in their studies of MCA-induced lung tumors in mice. Comparison of the preparation used at UCI with that used at the THRU indicated that, in both, the major proportion of MCA was as large crystals in the range of 50-300 μm, with a very small proportion in the range of 1-5 μm.

Table 3

$^{3}$H-BaP Metabolism by Lung and Liver Microsomes from MCA-Treated Rats Killed at 72 Hours or 7 Days Posttreatment

Values are picomoles of metabolites recovered from the incubation mixtures per mg protein after a 10 min incubation period, and are the average of duplicate samples. Blank values have been subtracted.

Part A: Rats Treated with 0.5 mg MCA and Killed 72 hours Later

| Tissue/Treatment | TLC Origin | 9,10-Diol | 7,8-Diol | 4,5-Diol | BaP-OH | Quinones |
|---|---|---|---|---|---|---|
| Lung/MCA | 21 | 43 | 69 | 68 | 401 | 170 |
| Lung/Vehicle | 0[a] | 1 | 0 | 3 | 16 | 0 |
| Lung/Sham | 0 | 0 | 0 | 3 | 17 | 0 |
| Lung/Anesthetized | 2 | 1 | 1 | 11 | 19 | 0 |
| Lung/Untreated | 5 | 1 | 2 | 2 | 21 | 0 |
| Liver/MCA | 642 | 870 | 637 | 572 | 986 | 817 |
| Liver/Vehicle | 74 | 79 | 74 | 113 | 93 | 350 |
| Liver/Sham | 83 | 77 | 71 | 122 | 88 | 382 |
| Liver/Anesthetized | 112 | 79 | 73 | 125 | 81 | 485 |
| Liver/Untreated | 73 | 100 | 85 | 126 | 306 | 305 |

Part B: Rats Treated with 1.0 mg MCA and Killed 1 Week Later

| Tissue/Treatment | TLC Origin | 9,10-Diol | 7,8-Diol | 4,5-Diol | BaP-OH | Quinones |
|---|---|---|---|---|---|---|
| Lung/MCA | 26 | 39 | 55 | 67 | 394 | 147 |
| Lung/Vehicle | 0 | 2 | 3 | 5 | 28 | 0 |
| Liver/MCA | 265 | 583 | 366 | 382 | 651 | 728 |
| Liver/Vehicle | 115 | 115 | 99 | 193 | 95 | 553 |

[a]A Value of "0" indicates that the radioactivity associated with the metabolite spot on the TLC plates was not significantly different from the blank values.

Table 4

Metabolism of $^{3}$H-BaP by Lung and Liver Microsomes from MCA-Treated Rats

Values are the percentage of input $^{3}$H-BaP converted to hydroxylated metabolites during a 10 min incubation at 37°C, and are the average of duplicate samples.

| | Days Posttreatment | | | |
|---|---|---|---|---|
| Tissue/Treatment | 2 Days | 7 Days | 14 Days | 21 Days |
| Lung/Untreated | 2.32 | 2.40 | 3.01 | 2.0 |
| Lung/Vehicle | 2.02 | 2.80 | 2.50 | 1.94[a] |
| Lung/MCA | 6.46 | 6.60 | 2.76 | 2.56 |
| Liver/Untreated | 17.7 | 19.4 | 22.3 | 18.8 |
| Liver/Vehicle | 14.9 | 16.9 | 19.1 | 15.8 |
| Liver/MCA | 54.8 | 27.7 | 19.3 | 18.1 |
| Blank: 1.89[b] | | | | |

[a]Single sample.

[b]Blank value obtained by incubation of $^{3}$H-BaP without microsomes. The blank has not been subtracted from the other values.

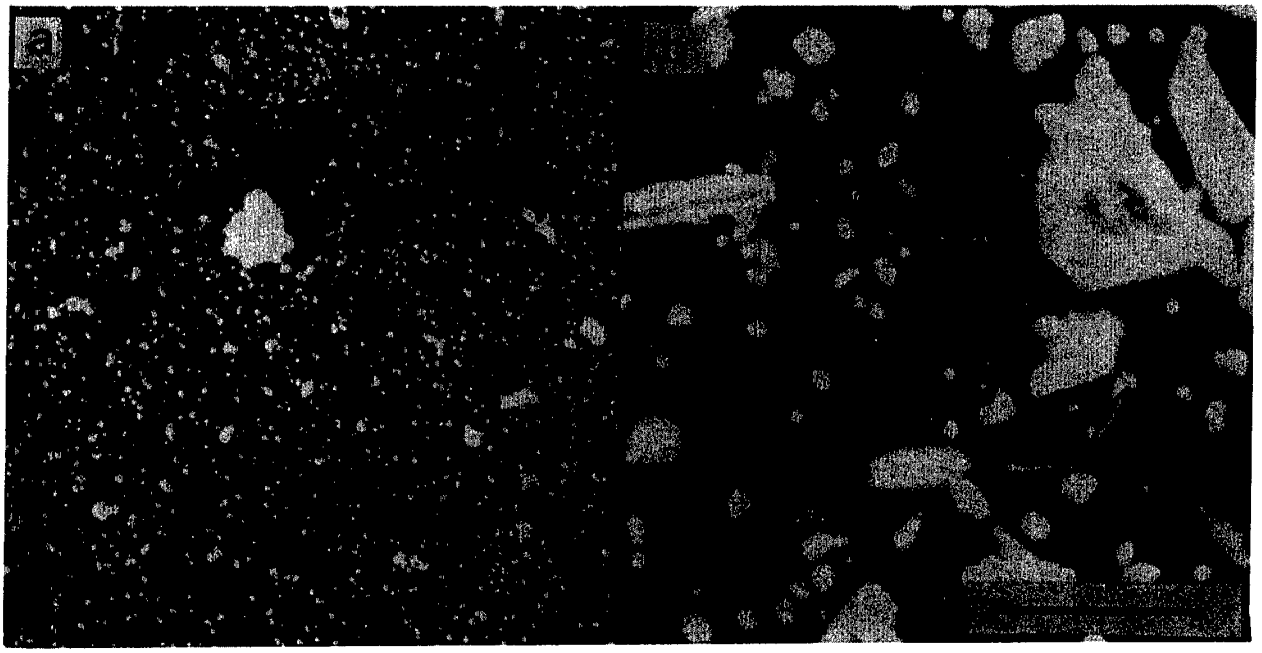


Figure 1. Darkfield photomicrographs of MCA crystals. a. Crystals obtained by sonication of MCA crystals as received from vendor (Sigma Chemical Company). b. Crystals obtained by recrystallization of MCA from benzene, followed by brief grinding in a mortar and pestle, Scale bar represents 50 µm.

At UCI, a series of experiments was undertaken using the large MCA particle preparation. The goals were to determine the persistence of enzyme induction after a single treatment, and the effect of repeated treatments with MCA in the manner used to induce lung tumors. In the first of these experiments, rats were inoculated once with 1.0 mg of MCA, and then sample animals killed at intervals up to 6 weeks posttreatment for measurement of $^{3}$H-BaP metabolism by lung and liver microsomes. The results are presented graphically in Figures 2 and 3. At 48 hr posttreatment, enzyme activity was high in both lung and liver microsomes. By 3 weeks, the activity of the liver enzymes had returned to control levels, but the lung enzymes were still elevated but slowly declining at the end of the six-week experiment.

A second experiment was done to obtain information on dose effects and to determine at what point the lung enzymes returned to the control level. Rats were inoculated once with either 0.1 mg or 1.0 mg MCA, or with the vehicle only. At intervals up to 11 weeks, sample animals were killed and lung and liver enzyme activities were measured. Figure 4 shows the results with the liver microsomes. At both doses, liver enzymes were induced at 2 days; by 14 days only the liver enzymes from rats treated with 1.0 mg MCA were above controls, and by 28 days there was no difference between the treated and control liver microsomes.

In the case of the lung microsomes, the enzyme levels were also increased at 48 hr (Figure 5). The enzyme activity declined with time posttreatment, but in the lungs of rats treated with 1.0 mg MCA remained above control levels for 8 weeks. In the rats treated with the lower dose, the enzyme activity had returned to control levels by 6 weeks. The level of enzyme activity in untreated rats declined over the course of the experiment as well. This latter observation has been made before, and is related to the age of the rats.

Effect of Intratracheal MCA on Cytochrome P-450 Spectra in Lung and Liver

Because the cytochrome P-450 content per mg protein of lung is 10-20 fold lower than liver, small amounts of contaminating materials can affect the absorbance spectra and thus make quantitation difficult. Therefore, we have not as yet been able to definitely correlate increased lung microsome cytochrome P-450 with increased $^{3}$H-BaP metabolic activity. However, comparison of the absorption maxima of the spectra of microsomes from MCA-treated and control rats clearly has shown an alteration in the composition of the cytochrome P-450 enzymes in both the liver and lungs of the MCA-treated rats. In both cases there was a shift of the absorbance maxima toward lower wavelengths. Figure 6 shows tracings of absorbance spectra for liver microsomal P-450 as measured in microsomes prepared 48 hours

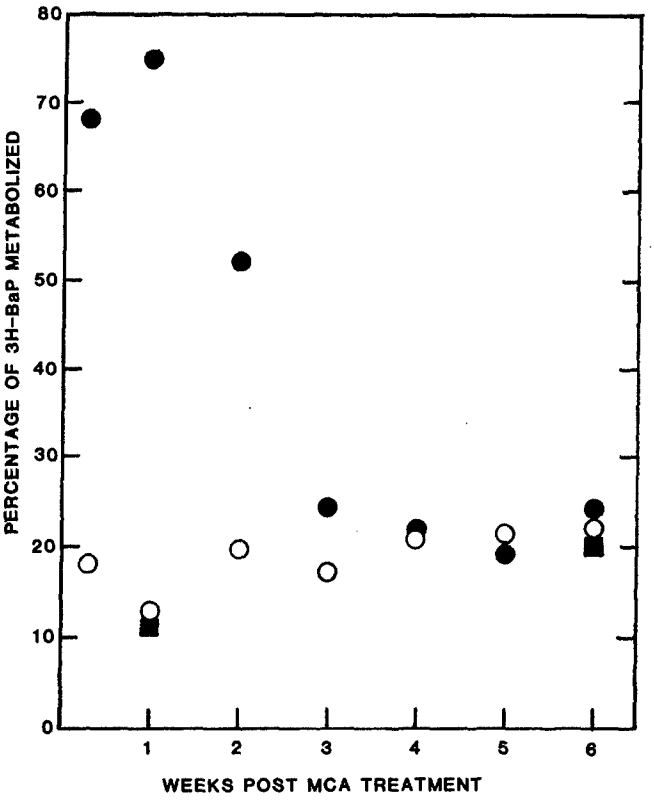


Figure 2. Metabolism of $^{3}$H-BaP by liver microsomes from MCA-treated rats. Blank values have not been subtracted. Open circles, vehicle control; closed circles, MCA treated; closed squares, untreated control.

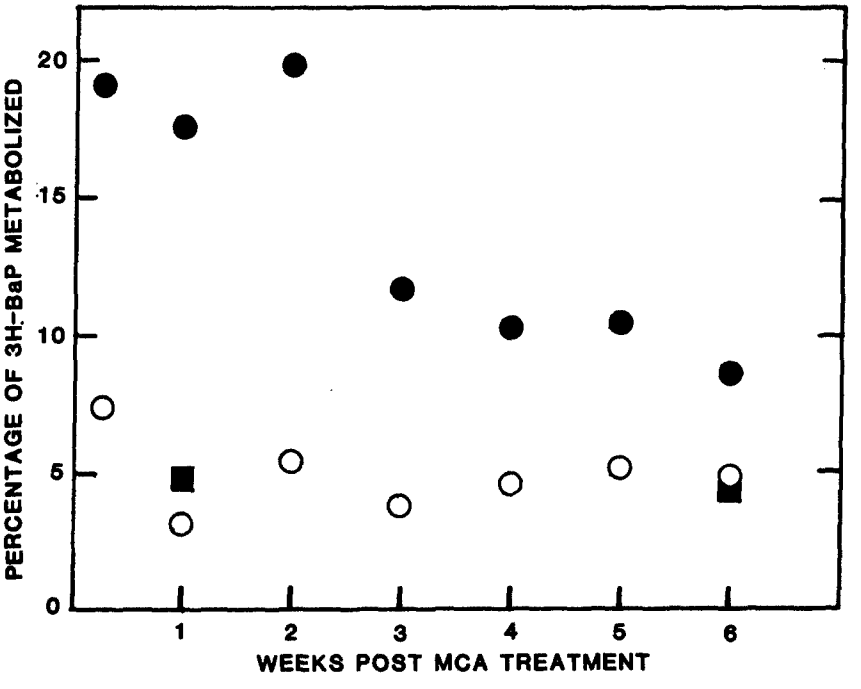


Figure 3. Metabolism of $^{3}$H-Bap by lung microsomes from MCA-treated rats. Blank values have not been subtracted. Symbols are as in Figure 2.

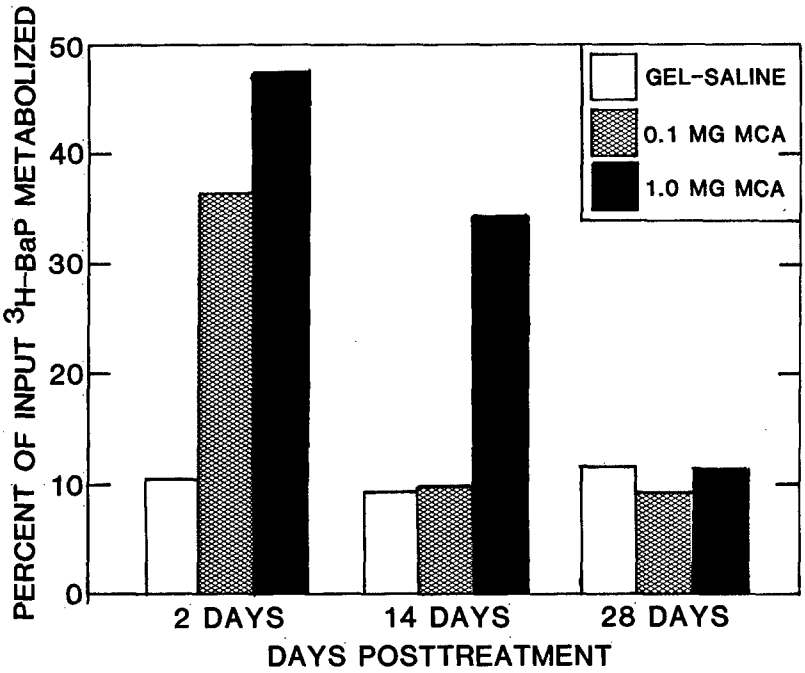


Figure 4. Metabolism of $^{3}$H-BaP by liver microsomes from rats treated with either 0.1 or 1.0 mg MCA. The values are the percent of input $^{3}$H-BaP converted to hydroxylated products, and are the average of duplicate samples.

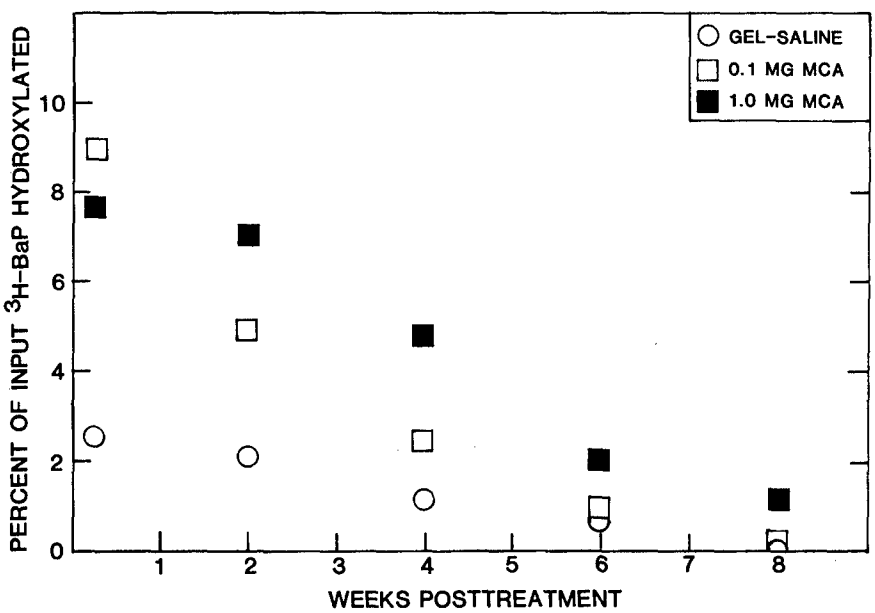


Figure 5. Metabolism of $^{3}$H-BaP by lung microsomes from rats treated with either 0.1 mg or 1.0 mg MCA. The values are the average of duplicate samples. Blank values have not been subtracted.

posttreatment. The shift in absorbance maximum is evident in the trace of the spectrum of the microsomes from MCA-treated rats (protein concentration was 2 mg/ml for both MCA-treated and control rats). The shift was also seen in the liver microsomes prepared one week after treatment, but was not present at 2 weeks or subsequently. The induction by chemical treatment of a species of cytochrome absorbing at 448 nm has been reported in many animal systems. The results found in this experiment show that this type of enzyme (cytochrome P-448 or $P_1$-450) can be induced in the liver when the chemical (MCA) is given by intratracheal inoculation.

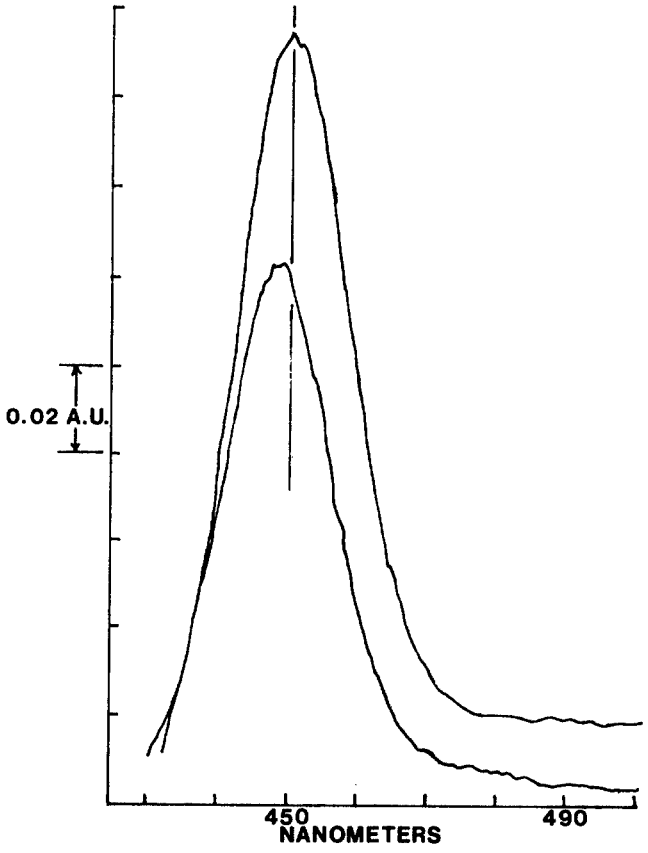


Figure 6. Difference absorbance spectra of microsomal cytochrome P-450 obtained with liver microsomes from vehicle control or MCA-treated rats at 48 hr posttreatment. Upper curve, control; lower curve, MCA-treated. The methods are described in the text.

Spectral analysis of microsomes prepared from the lungs of the treated rats also indicated the appearance of a species of cytochrome P-450 not normally found in great amounts. Figure 7 shows tracings of difference spectra obtained with lung microsomes prepared at 2 days posttreatment. The normal peak absorbance for lung cytochrome P-450 is about 453 nm. The shift toward lower wavelengths is evident.

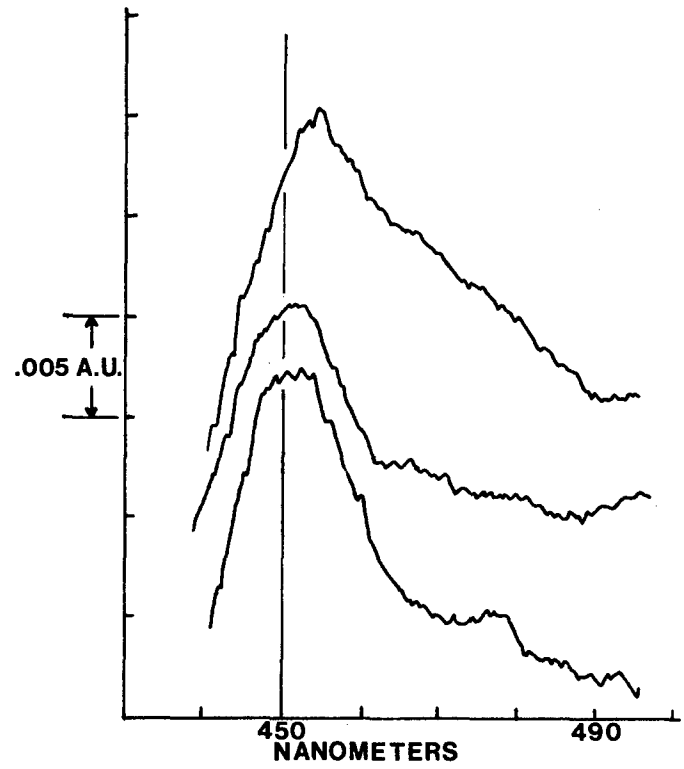


Figure 7. Difference absorbance spectra of microsomal cytochrome P-450 obtained with lung microsomes prepared at 48 hr posttreatment. Upper curve, vehicle control; middle curve, 1.0 mg MCA; lower curve, 0.1 mg MCA. The methods are described in the text.

The possibility that the alteration in the relative amounts of P-450 species might change the proportions of the metabolites of $^{3}$H-BaP produced by lung microsomes was examined. Comparison of the percentage yields of the major metabolites produced by lung microsomes from control, 0.1 mg, and 1.0 mg MCA-treated rats showed no difference. The data are summarized in Table 5, which contains results from rats killed at 48 hr posttreatment. The yields of all major metabolites were increased to approximately the same extent in the MCA-treated rats.

Table 5

Comparison of Relative Yields of Metabolites of $^{3}$H-BaP with Lung Microsomes from MCA-Treated and Control Rats

Young rats (ca. 100 gm) were inoculated intratracheally with either 0.1 or 1.0 mg MCA and killed 48 hr later. The yields were calculated as picomoles per mg protein during a 10 min incubation period at 37°C, and are the average of duplicate samples. The fraction of the total yield appearing as each metabolite is indicated in parentheses.

| Metabolite | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Vehicle | | 0.1 mg MCA | | 1.0 mg MCA | |
| TLC Origin | 9.0 | (0.03) | 40 | (0.03) | 38 | (0.03) |
| 9,10-Diol | 40 | (0.13) | 147 | (0.10) | 151 | (0.12) |
| 7,8-Diol | 45 | (0.14) | 182 | (0.12) | 184 | (0.14) |
| 4,5-Diol | 47 | (0.15) | 166 | (0.11) | 165 | (0.13) |
| Monohydroxy-BaP | 177 | (0.56) | 1010 | (0.65) | 762 | (0.59) |

MCA-Induced Sister Chromatid Exchanges (SCEs) in Lung Cells

An increased incidence of SCEs has been shown in rat lung cells exposed either in vivo or in vitro to MCA. For in vitro exposure, lung cell cultures, prepared as described under Methods, were incubated for 24 hr in medium containing MCA at 1 µg/ml or 10 µg/ml. SCE incidence was measured as described and the results are given in Table 6. At both concentrations, a very significant increase in SCEs was found. This result indicates that the lung cells are capable of metabolizing MCA to reactive intermediates which can cause chromosomal damage.

In vivo treatment with MCA in the manner described also produced an increase in SCEs in lung cells. After a single intratracheal treatment with MCA, lung cell cultures were prepared from sample rats up to 6 weeks posttreatment. The one treatment with 1.0 µg MCA resulted in a persistent elevation of SCEs through at least the sixth week posttreatment (Table 7). At the lower dose, the incidence of SCEs was not different from the controls except for the 4 week sample. Cultures prepared from rats at times longer than 6 weeks posttreatment have not yet been analyzed.

Table 6

In Vitro Induction of SCEs in Primary Cultures of Rat Lung Cells by MCA

Primary cultures of lung cells were established and treated with MCA as described in the text. Values are the mean number of SCEs per chromosome ± 1 S.D. The number of cells scored is indicated in parentheses.

| Treatment | Complete Metaphases | Incomplete Metaphases |
|---|---|---|
| Vehicle (0.1% DMSO) | 0.627 ± 0.15 (21) | 0.744 ± 0.25 (32) |
| 1 µg MCA/ml | 1.136 ± 0.31[a] (25) | 1.098 ± 0.32[a] (62) |
| 10 µg MCA/ml | 1.157 ± 0.36[a] (19) | 1.216 ± 0.30[a] (81) |

[a]All values for MCA-treated cultures are significantly above controls ($p < 0.01$, student's one-tailed "t" test).

Table 7

Incidence of SCEs in Primary Cultures of Lung Cells from MCA-Treated Rats

Values are SCEs per chromosome ± 1 S.D. the number of cells scored is in parentheses.

| Time Post-Treatment | Treatment | | |
|---|---|---|---|
| | Vehicle | 0.1 mg MCA | 1.0 mg MCA |
| 48 hr | 0.611 ± 0.18 (71) | No Data | 1.248 ± 0.32 (73)[a] |
| 2 wk | 0.683 ± 0.25 (99) | 0.731 ± 0.18 (22)[b] | 0.957 ± 0.31 (41)[a] |
| 4 wk | 0.513 ± 0.15 (98) | 0.655 ± 0.20 (98)[a] | 0.686 ± 0.21 (89)[a] |
| 6 wk | 0.578 ± 0.20 (100) | 0.624 ± 0.22 (66)[b] | 0.907 ± 0.28 (58)[a] |

[a]Significantly above the vehicle control ($p<0.005$).
[b]Not significantly different from vehicle control.

## DISCUSSION AND CONCLUSIONS

The above experiments have shown that intratracheal inoculation with suspensions of MCA crystals will induce in lung and liver the enzymes involved in metabolic activation and detoxification of polynuclear hydrocarbons such as BaP and MCA. They further have shown that the induced enzymes remain elevated longer in the lung

than in the liver. A tentative conclusion from these observations is that the MCA is sequestered in the lung (e.g., in macrophages) and is only slowly cleared from the lung. These suggestions are supported by the experiments using small or large particles of MCA. With the small particle preparation, the lung enzymes had returned to normal levels by 14 days posttreatment, but with the larger particles, the enzyme levels were above controls up to 8 weeks. More objective measurements of the clearance of MCA from the lung are planned for the upcoming year.

In the experiments conducted at the THRU to establish the dose response for tumor initiation by MCA, rats were inoculated at biweekly intervals. At the highest dose (25 mg total, 5 mg/dose) some rats had developed lung tumors by the time of the 5th dosing. The results reported here with the large MCA crystals indicated that the enzyme levels in the lung would be high at the times of the repeated treatments, thus favoring the chances for formation of high concentrations of the carcinogenic intermediates of MCA. In future experiments, the metabolism of MCA by lung microsomes from treated rats will be examined in order to determine whether high levels of reactive intermediates are indeed formed in the lung.

Evidence for persistent chromosomal damage by MCA in the lung has been found in the studies of SCE incidence in primary cultures of lung cells. Reports of others (Stetka et al.,1978) also have indicated that chemical carcinogens can produce lasting chromosomal damage. The persistence of elevated SCE incidence may also result from the slow clearance of MCA from the lung, in which case lung cells would be continuously exposed to the active metabolites of MCA. Resolution of these alternatives will be the subject of future experiments in which rats will be treated with either small MCA particles, which are cleared rapidly, or with large MCA particles, which are cleared more slowly. Measurement of SCE incidence at times after treatment will show the relationship between clearance of MCA and induction of SCEs.

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